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Introducing mothur: Open Source, Platform-independent, Community-supported Software for Describing and Comparing Microbial Communities

Running title: Introducing mothur
Appropriate Section: Methods

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Summary

mothur aims to be a comprehensive software package that allows users to use a single piece of software to analyze community sequence data. It builds upon previous tools to provide a flexible and powerful software package for analyzing sequencing data. As a case study, we used mothur to trim, screen, and align sequences, calculate distances, assign sequences to OTUs, and describe the α- and β-diversity of eight marine samples previously characterized by pyrosequencing of 16S rRNA gene fragments. This analysis of more than 222,000 sequences was completed in less than 2 hours using a laptop computer.

Key words: metagenomics, bioinformatics, next-generation sequencing

Since Pace and colleagues (18) outlined the culture-independent framework for sequencing 16S rRNA gene sequences in 1985, microbial ecologists have experienced an exponential improvement in the ability to sequence not only this primary phylogenetic marker but also numerous functional genes from diverse environments. Twenty-five years later, there are over 10^6 rRNA gene sequences deposited in public repositories such as GenBank and the number of sequences continues to double every 15-18 months (http://www.arb-silva.de/news/view/2009/03/27/editorial/). The development of pyrosequencing technologies has enabled the Human Microbiome Project (29), International Census of Marine Microbes (ICoMM; http://icomm.mbl.edu), and individual investigators to collectively amass over 10^9 16S rRNA gene sequences tags since 2006. Because of this development in sequencing technology, individual studies have shifted from sequencing 10^1-10^2 sequences from multiple samples (e.g. 2, 16) to sequencing 10^4-10^5 sequences from multiple samples (e.g. 27, 28). These impressive statistics are indicative of the excitement the field enjoys over relating changes in microbial community structure with changes in ecosystem performance.

Advances in computational tools have improved our ability to address ecologically-relevant questions. Because of the development of tools including ARB (13), DOTUR (22), SONS (23), LIBSHUFF (25, 26), UniFrac (11, 12), AMOVA and HOMOVA (15, 21), TreeClimber (24), and rRNA-specific databases (3, 4, 20), microbial ecology has progressed from being a descriptive to an experimental endeavor. Although these tools have been widely successful, a number of limitations will affect their use as sequencing capacity increases and studies become more complex. First, for ease of use many of the rRNA-specific databases have online tools including aligners, classifiers, and analysis pipelines; however, these tools allow a limited set of generic analyses and we must begin to question whether transferring gigantic datasets across the internet for analysis is a sustainable practice. Second, much of the existing software was developed for analyzing 10^2 to 10^4 sequences. As the number of sequences expands it is essential that existing software be re-factored to use more efficient algorithms. In addition,

72 although the use of scripting languages such as Perl and Python have been useful for the online
73 analysis of small datasets, they are relatively slow compared to code written in C and C++.
74 Finally, the boutique nature of the existing tools has limited their integration and further
75 development. One consequence of this is that the generation of field-wide analysis standards
76 have not been developed making it difficult to perform meta-analyses. As sequencing capacity
77 increases and our research questions become more sophisticated, it is critical that the software
78 be flexible and easily maintained.

79 **Introducing mothur.** To overcome these limitations, we have developed a single
80 software platform, mothur (Table 1). mothur implements the algorithms implemented in
81 previous tools including DOTUR, SONS, TreeClimber, LIBSHUFF, ∫-LIBSHUFF, and UniFrac.
82 Beyond the implementation of these approaches, we have incorporated additional features
83 including: (i) over 25 calculators for quantifying key ecological parameters for measuring α- and
84 β-diversity; (ii) visualization tools including Venn diagrams, heat maps, and dendrograms; (iii)
85 functions for screening sequence collections based on quality; (iv) a NAST-based sequence
86 aligner (5); (v) a pairwise sequence distance calculator; and (vi) the ability to either call
87 individual commands from within mothur, using files with lists of commands (i.e. batch files), or
88 directly from the command line provide for greater flexibility in setting up analysis pipelines.

89 **Object oriented, responsive, free, and platform-independent.** mothur is written in
90 C++ using modern object oriented programming strategies (17, 19). Design patterns are used
91 extensively to improve the maintenance and flexibility of the software (7). Since releasing the
92 first version of mothur in February 2009, we have made use of an iterative release design
93 model. This means that instead of releasing mothur once a year with many modifications, we
94 release smaller updates to mothur throughout the year. The advantage to this approach is the
95 ability to more quickly address bugs, incorporate user suggestions, and get new features to
96 users. By making mothur an open source software package under the GNU General Public

License (http://www.gnu.org/licenses/gpl.html), the software is free and open to modification by other investigators developing their own analysis methods. mothur is available from the project website (http://www.mothur.org) as a Windows-compatible executable or as source code for compilation in Unix/Linux or Mac OS X environments.

Open documentation and support. Extensive community-supported documentation and support are available through a MediaWiki-based wiki (http://www.mediawiki.org) and a phpBB-based discussion forum (http://www.phpbb.com). The wiki format serves two important functions. First, it is a source of documentation that users are free to read, edit, and expand to help themselves and others understand the theory and implementation behind the commands provided in mothur. For example, the wiki-page describing each calculator includes manual calculations. Numerous undergraduate and graduate courses have used these example calculations to improve their students' numeracy. Second, users are encouraged to create pages describing how they used the software to analyze a set of data as a medium for teaching others the diverse ways that one can design experiments and analyze their data. These "example workflows" include the original data, commands, and commentary from unpublished and published studies (e.g. 1, 8, 9). The discussion forum allows users to ask questions that anyone can answer and the forum allows users to suggest improvements to the software.

Example workflow: The Ocean's Rare Biosphere. Although mothur is fully capable of analyzing traditional clone-based sequences, here we demonstrate the ability of mothur to efficiently analyze a pyrosequencing dataset. Sogin and colleagues seminal 2006 study that outlined the use of pyrosequencing in microbial ecology studies obtained 216,243 high quality sequence reads from the V6 region of the 16S rRNA gene from 8 samples (27). They obtained six-paired samples from the meso- and bathypelagic realms from three sites in the North Atlantic Deep Water loop and two samples from diffuse hydrothermal vent fluids near the site of an eruption in the Axial Seamount in the northeast Pacific Ocean (Fig. 1). Their analysis primarily considered their inability to exhaustively sample the biodiversity of sites in spite of

record sequencing depths. The sequence data were obtained from http://jbpc.mbl.edu/research_supplements/g454/20060412-private/ and we used the February 2, 2008 version of the dataset. These data differ from those described in the original publication because the data processing algorithms internal to the GS20 machine were updated; therefore, it is not possible to make a direct comparison to the findings of the original analysis. Although these data were already trimmed and sorted into individual files for each sample, mothur has the capacity to generate these files from the FASTA-formatted sequence file generated by a sequencer. Furthermore, mothur has a number of functions for performing hypothesis tests, but here we will focus on operational taxonomic unit (OTU)-based methods of describing and comparing communities.

mothur makes several improvements that allow users with modest computing resources to analyze large datasets. Most significant are the ability to only analyze the unique sequences in a dataset, but retain information about the number of times each sequence was observed and the use of sparse matrices that only represent distances smaller than a user-specified cutoff. Using a PHYLIP-based approach would have required approximately 145 GB to represent 2.3×10^{10} distances. Our improvements resulted in an 18.9-MB file containing 5.2×10^{5} pairwise distances that were smaller than a 0.10. The only mothur-imposed limit is the number of distances that can be processed, which is 2^{64}. The more likely limitation will be the amount of RAM available on the user's computer. With the reduced memory requirement also comes significantly improved processing speed. Considering most computers have multiple processors, users can obtain further increases in speed by utilizing the parallelization features provided in the alignment and distance calculation commands.

mothur can cluster sequences using the furthest neighbor, nearest neighbor, or UPGMA algorithms (22). The ability to let the data speak for themselves in determining OTUs is advantageous compared to database-based approaches that can form clusters, in which sequences are similar to the same database sequences, but not to each other. Furthermore,

mothur uses the approach employed in DOTUR where OTUs are defined for multiple cutoffs up to the distance threshold so that alternative OTU definitions can be compared. For example, using the furthest neighbor algorithm, we clustered sequences into OTUs up to a distance threshold of 0.10 and observed 13,202, 11,317, and 7,971 OTUs at cutoffs of 0.03, 0.05, and 0.10 distance units. A similar type of analysis using the approach used in programs such as CD-HIT would limit the user to a nearest neighbor-based approach and the user would need to run the program for each distance level that they were interested in (10).

By inputting a file that maps each sequence to a sample identifier, the clusters could be parsed to perform α-diversity analyses. First, we calculated the richness and diversity of the 8 samples at OTU cutoffs of 0.03, 0.05, and 0.10 distance units using the number of observed OTUs, Chao1 estimated minimum number of OTUs, and a non-parametric Shannon diversity index (Table 2). Second, we calculated rarefaction curves for the eight samples for a 0.10 distance cutoff (Fig. 2); the original Sogin analysis built rarefaction curves using frequencies acquired from a database-based OTU assignment analysis. Interestingly, mothur calculated the coverage of these samples to be between 0.94 and 0.98, yet the rarefaction curves continued to climb with increasing sequencing effort. These types of analysis were the extent of the α-diversity measurements performed in the original Sogin analysis and each sample required up to 4 days to complete on a Quad Opteron 875 2.2 GHz series Dual Core machine with 28 GB of RAM (Sue Huse, personal communication). The analysis described in this manuscript – from aligning of sequences through β-diversity analyses – required less than 2 hrs using a MacBook Pro laptop with 2 GB RAM and using only one of the 2.0 GHz duo processors.

Due to software limitations, it was not possible to assess the β-diversity of the samples in the original Sogin analysis. With the software improvements implemented in mothur, we were able to transform the original OTU information into heatmaps, Venn diagrams, and dendrograms (Fig. 1) to describe the similarity in membership and structure of the 8 samples. Several

interesting observations can be made from this analysis. First, although the dendrograms generated using the Jaccard coefficient and the Θ_{YC} community structure similarity coefficient have similar topologies, the terminal branch lengths of the Jaccard coefficient dendrogram are considerably longer for samples 53R, 55R, 115R, and 137. This is interesting because it indicates that while these samples have considerably different memberships (Jaccard), the relative abundance of the shared OTUs is similar. Thus, the differences between the communities are likely found in the rarer OTUs. Second, the two diffuse hydrothermal flow samples clearly cluster away from the others. This is intuitive because of the considerable differences in temperature and chemistry. Third, the only available piece of meta-data that explains the clustering of the seawater samples is extreme depth; the deepest sample, 112R, clearly clusters away from the other seawater samples and was taken 2,411 m deeper than any of the other samples. Considering this was the only sample taken at such an extreme depth, additional sampling is required to have confidence in such a correlation.

Looking forward. The development of computational tools to describe and analyze microbial communities is in a "Red Queen"-type race where advances in computational power are met with expansions in sequencing capacity and vice versa. As the length and number of reads multiply, data analysis resources must meet the challenge. Although mothur goes a long ways to making data analysis efficient, flexible, and simple, the analyses are by no means trivial and researchers must take care to ensure that their experiments are well designed, thought-out and that their results are biologically plausible. The field of microbial ecology is experiencing an amazing revolution where we can now design experiments with sophisticated experimental designs. Tools such as mothur open new possibilities so that the primary limitation is our imagination.

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Figure 1. Description and comparison of the eight samples analyzed by Sogin et al. (27). The dendrogram to the left represents the similarity of the samples based on the membership-based Jaccard coefficient calculated using Chao1 estimated richness values. The dendrogram on the right represents the similarity of the samples based on the structure-based Θ_{YC} coefficient. The distance from the tip of the dendrogram to the root is 0.50 for both trees.

Figure 2. Rarefaction curves describing the dependence of discovering novel OTUs as a function of sampling effort for OTUs defined at a 0.10 distance cutoff. The curves for FS312 and FS396 climb to 3,095 and 2,804 OTUs after sampling 54,894 and 80,769 sequences, respectively.

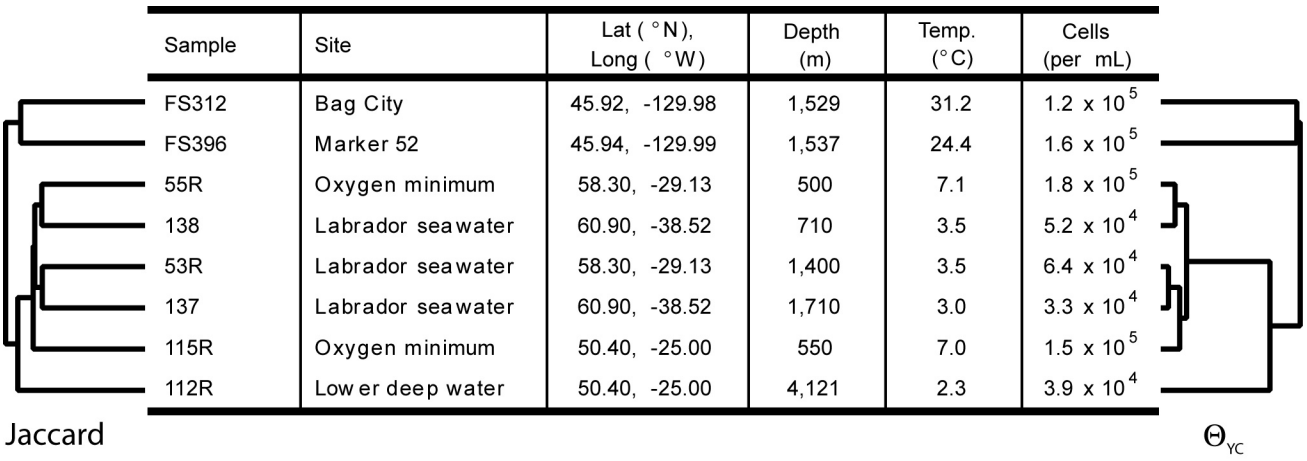
288 **Table 1. Features from pre-existing software that have been integrated into mothur. In**
289 **all cases, modifications have been made to the implementation of the algorithms for**
290 **greater flexibility, speed, and resource utilization.**

Existing tool	Description	Implementation in mothur	Ref.
Pyrosequencing pipeline (RDP)	Online tool that trims and deconvolutes sequences using user-supplied data	Stand-alone implementation; increased speed; greater flexibility; additional screening options	(3)
NAST, SINA, and RDP Aligners	Online tools that align user-supplied sequences to specific databases	Stand alone implementation; can utilize multiple processors; increased speed; greater flexibility; open source	(3-5, 20)
DNADIST	Calculates pairwise distances between sequences (does not penalize for gaps)	Can utilize multiple processors; more efficient use of RAM; various ways to penalize gaps	(6)
DOTUR AND CD-HIT	Assigns sequences to OTUs, constructs sampling curves, and estimates richness and diversity	More efficient clustering; requires less memory; additional calculators; greater flexibility	(10, 22)
SONS	Calculates estimates of the fraction and richness of OTUs shared between communities	Generates dendrograms, heatmaps, and venn diagrams; additional calculators; greater flexibility	(23)
∫-LIBSHUFF	Uses the Cramer-von Mises statistic to test whether two communities have the same structure	No longer need a sorted distance matrix; can specify pairwise comparisons	(25, 26)
TreeClimber	Uses a parsimony-based test to determine whether two or more communities have the same structure	Greater flexibility; can specify pairwise comparisons	(14, 15, 24)
UniFrac	Compares the phylogenetic distance between communities to detect differences in community structure	Stand alone implementation; greater flexibility; can input bootstrap trees	(12)

291 **Table 2. Measures of α-diversity for the samples characterized by Sogin et al. (27) for**

292 **three OTU definitions.**

Sample	Reads	0.03			0.05			0.10		
		OTU	Chao	H'	OTU	Chao	H'	OTU	Chao	H'
53R	12,725	1,599	3,222	5.29	1,420	2,622	5.19	1,053	1,733	4.81
55R	9,848	1,469	2,994	5.54	1,302	2,496	5.43	962	1,741	5.03
112R	15,057	2,258	5,189	5.91	2,032	4,282	5.79	1,584	2,992	5.44
115R	16,181	1,749	3,600	5.31	1,552	3,088	5.21	1,135	1,919	4.83
137	13,831	1,425	2,687	5.44	1,295	2,430	5.36	989	1,645	5.07
138	12,938	1,425	2,542	5.24	1,253	2,131	5.14	957	1,479	4.81
FS312	54,894	4,371	10,691	5.23	3,948	9,259	5.16	3,095	6,409	4.94
FS396	80,769	4,359	10,208	4.67	3,806	8,609	4.60	2,804	5,437	4.42



Sample	Site	Lat (°N), Long (°W)	Depth (m)	Temp. (°C)	Cells (per mL)
FS312	Bag City	45.92, -129.98	1,529	31.2	1.2×10^5
FS396	Marker 52	45.94, -129.99	1,537	24.4	1.6×10^5
55R	Oxygen minimum	58.30, -29.13	500	7.1	1.8×10^5
138	Labrador seawater	60.90, -38.52	710	3.5	5.2×10^4
53R	Labrador seawater	58.30, -29.13	1,400	3.5	6.4×10^4
137	Labrador seawater	60.90, -38.52	1,710	3.0	3.3×10^4
115R	Oxygen minimum	50.40, -25.00	550	7.0	1.5×10^5
112R	Lower deep water	50.40, -25.00	4,121	2.3	3.9×10^4